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To:	Examiner Christopher Yaen Art Group 1642	**From:**	Leonard R. Svensson/Susan W. Gorman
Fax:	703-308-4242	**Pages:**	33 (including cover sheet)
Phone:	703-305-3586	**Date:**	September 17, 2003
Your Ref.:	09/889,300	**Our Ref.:**	0147-0229P
Re:	Materials for Interview on 09/19/2003	**CC:**	

☐ **Urgent** ☐ **For Review** ☐ **Please Comment** ☐ **Please Reply** ☐ **Please Recycle**

Comments:

Dear Examiner Yaen,

Please find attached the following materials for the interview on Friday, September 19 at 3:30 p.m.:

- ☐ **UNOFFICIAL** Outline of Interview
- ☐ **UNOFFICIAL** Proposed Amended Claims
- ☐ **UNOFFICIAL** Declaration of Hans Loibner dated September 9, 2003
- ☒ **UNOFFICIAL** Declaration of Hans Loibner dated July 17, 2002.

Best regards,

Leonard R. Svensson
Susan W. Gorman

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	: Yaen; Christopher H
Group Art Unit	: 1642
Applicants	: Eckert, Helmut et al.
Serial No.	: 09/889,300
Filed	: September 13, 2001
For	: USE OF ANTIBODIES FOR THE VACCINATION AGAINST CANCER

#11
KG
9/25/03

Hon. Assistant Commissioner
For Patents
Wahington, D.C. 20231

DECLARATION UNDER 37 C.F.R. § 1.132 of HANS LOIBNER

I, Hans Loibner, hereby declare and state as follows:

1. I am one of the co-inventors of the subject matter of the above-identified application.
2. I am currently Chief Executive Officer of Igeneon Krebs-Immuntherapie-Forschungs-und Entwicklungs-AG, a research based biotechnology company. Prior to that I was working as head of R&D of cancer vaccines for more than 15 years. My Curriculum vitae is enclosed as Exhibit 1.
3. I have published over 15 scientific papers in the field of cancer research, a list of publications is enclosed as Exhibit 2.
4. I have read the Office communication of May 8, 2002, paper number 8, in the above-identified application and the documents cited therein. I make this declaration to make of record supplemental results, which further demonstrate the effectiveness of the methods described in the above-identified application.

5. Specifically I make this declaration to present the following experimental data generated in my laboratory under my direction and supervision:

Example A

Methods:

Goats have been immunized with two different antibody preparations:

a. HE2 Antibody as disclosed in the above-identified application
b. 73-3 Antibody (anti-EpCAM, Hybridoma 1986 (5) Suppl1: 79-86, Exhibit 3)

Two goats were immunized by intradermally injecting of 1.5 mg of each antibody preparation using Freunds Complete Adjuvant. Following initial vaccination, a booster injection was given on day 8 using Freunds Incomplete Adjuvant. A second booster injection was given day 29, however, with no adjuvant. Binding of antibodies to EpCAM-positive tumor cells (KATO III gastric cancer cell line) was tested in pre-serum and in serum of day 54. As a control EpCAM negative cells (WM9 melanoma cells) were used. Testing was performed employing a cell-ELISA as described in the above-identified application.

Results:

The results are illustrated by the figure of Exhibit 4: Purified antibodies produced after vaccination with HE2 was called SCV106, antibodies produced after vaccination with 73-3 antibody was called "goat anti-id 73-3". "Goat IgG" purified from non-vaccinated goats, was used as a control.

Goat immunosera contained immunoglobulins that bind to EpCAM positive KATO cells. There was no binding to WM9 control cells. In pre-serum there were no such antibodies binding to the KATO cells. It was thus proven that vaccination with the anti-EpCAM antibodies HE2 and 73-3, which bind to different epitopes of the EpCAM molecule, induced again antibodies against the EpCAM molecule.

Example B

Methods:

Rhesus monkeys have been immunized with two different antibody preparations:

a. HE2 Antibody as disclosed in the above-identified application
b. KS1/4 Antibody (anti-EpCAM, Cancer Research 44, 681-687 (1984) Exhibit 5)

Two rhesus monkeys were immunized by intradermal injections of each of the antibody preparations (0.5 mg) at day 1, 15, 29 and 57. Blood samples were tested for antibody titers on day 0 (preserum) and day 71. Antibodies were isolated by immunoaffinity chromatography using sequential immunoaffinity chromatography. The first column contained the immunizing agent as ligand, i.e. antibody HE2 or KS1/4, to isolate immunoglobulins resulting from the immune response. The second column contained recombinant EpCAM as ligand. Thus, the EpCAM specific immune response was isolated and quantified.

Results:

The results are provided in the following table: Presera contained no specific antibodies. Specific antibodies, however, were isolated from the immune sera of both groups. Thus, vaccination with both anti-EpCAM antibodies induced an antibody titer specific for EpCAM positive tumor cells.

Table:

Immunizing antibody	Immune response specific for EpCAM (μg Immunoglobulin/ ml serum)
0.5 mg HE2	53
0.5 mg KS1/4	110

6. It is my belief that based on the data showing that the method as disclosed in the specification provides a valid teaching about the production of a cancer vaccine that exhibits similar properties as the vaccine based on the HE2 antibody. Antibodies used to manufacture a vaccine according to the present application were 73-3, KS1/4 and HE2, each of them binds to different epitopes of EpCAM. According to the disclosure of the present application it was possible to provide suitable vaccines based on these antibodies that proved to provoke an immune response directed against EpCAM positive tumor cells.
7. One of skill in the art learning from the specification that an anti-EpCAM antibody like HE2 is a suitable antigen in cancer vaccines, would be able to easily manufacture a vaccine based on any anti-EpCAM antibody.
8. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of this application or any patent issuing thereon.

HANS LOIBNER, Ph.D.

Signed this 17 day of
July, 2002
At Vienna, Austria

US 09/889,300
Declaration of HANS LOIBNER
Exhibit 1

Curriculum Vitae
Hans Loibner, Ph.D.

Date of Birth:	October 16, 1947
Place of Birth:	Vienna, Austria
Citizenship:	Austria
Address:	Igeneon Krebs-Immuntherapie Forschungs- und Entwicklungs-AG Brunner Strasse 59, A-1230 Vienna, Austria Tel: + 43 1 869 8050 245 Fax: + 43 1 869 8050 289
Home address:	Heimgasse 23, A-1238 Vienna, Austria Tel: + 43 1 888 7615
Family status:	Divorced, 4 children
Education:	Primary school High school University Vienna: Chemistry
1975 - 1977	Thesis at Department of Organic Chemistry, Prof.Dr.E.Zbiral, University Vienna, in synthetic organic chemistry
1977	Ph.D.
1977 - 1979	**Postdoctoral fellow** at Sandoz Forschungsinstitut (SFI): Chemical synthesis of new aminoglycoside antibiotics with improved properties (active against resistant strains and less toxicity); Creation of an efficient system for preparative medium pressure high performance liquid chromatography.
1979 - 1984	**Head of a chemistry laboratory** at SFI: Synthesis of aminoglycoside antibiotics as above; synthesis of immunostimulants.
1981-1984	**Head of working group "Aminoglycoside-Antibiotics"** Coordination of all activities in chemistry, biology and toxicology for characterization of novel aminoglycoside antibiotics and identification of development candidates.
1984 - 1986	**Head of Department "Molecular Enzymology"** at SFI: Main tasks: • Mechanism based strategies for discovery and characterization of substances for immunostimulation • Enzymatic preparative synthesis and biological characterization of lipid-A analogs • Enzymological and cellular tests for assessment of macrophage stimulation • Protein chemistry, protein purification and -analytics

1986 - 1989 **Head of Department "Antibodies"** at SFI:
Main tasks:
- Discovery, profiling and development of antibody-based immunotherapies against cancer, in particular tumor specific monoclonal antibodies for passive immunotherapy and anti-idiotypic antibodies for therapeutic vaccination
- Generation and characterization of new monoclonal antibodies for all research purposes of the institute
- Fermentation of bacteria as well as of mammalian cells for production of proteins
- Protein purification and -analytics; automated peptide synthesis.

1987 - 1994 **Head of international project teams**
Responsibility for coordinated international preclinical and in particular clinical development of projects in specific cancer immunotherapy
(ABL 364: monoclonal antibody for passive immunotherapy, SCV 106: anti-idiotypic antibody vaccine for therapeutic vaccination), together with internal departments and involved external clinical centers.

1990 - 1994 **Head of Department "Special Projects"** at SFI:
Main tasks:
- Assessment of immunological and serological parameters of cancer patients in the context of clinical studies with cancer immunotherapy approaches developed at SFI
- Generation and preclinical profiling of new projects in specific cancer immunotherapy

1995 **Head of Department Genetics** at SFI
Main tasks:
- Establishment of highly automatized methods for comprehensive analysis of expressed genes as novel tool for identification of molecular targets for disease intervention in all disease areas of interest for Sandoz Pharma
- Shaping of the collaboration with Prof.Lehrach, MPI Berlin regarding Fingerprinting analysis of expressed genes
- Responsibility for preclinical development of an anti-idiotypic antibody vaccine against epithelial cancer (MMA 383) generated at SFI in the context of an international project team

1996 **Head of MMA 383 Support Group** at SFI
Main task:
Support of further development of the anti-idiotypic antibody vaccine MMA 383, aiming at initiation of clinical proof of concept trials 1/1997

1997 - 1999 **Head of Oncology Group Vienna** at Novartis Forschungsinstitut
Main tasks:
Support of the anti-idiotypic antibody vaccine MMA 383 as above. Basic research in the area of active specific immunotherapy.

1999 - 2001 **Head of Igeneon GmbH**
Development of cancer vaccines and immunotherapies

2001 - **CEO, Igeneon Krebs-Immuntherapie Forschungs-und Entwicklungs-AG**

US 09/889,300
Declaration of HANS LOIBNER
Exhibit 2

List of publications
Hans Loibner, Ph.D.

Full papers and book chapters

Increased expression of the blood group related LeY antigen on synovial fluid granulocytes of patients with inflammatory joint diseases
M. Dettke, G. Palfi, E. Pursch, E. Leeb, J. Smolen and H. Loibner
Rheumatology (Oxford). 2001 Sep;40(9):1033-7.

Activation-dependent expression of the blood group related Lewis Y antigen on peripheral blood neutrophils
M. Dettke, G. Palfi and H.Loibner
J Leukoc Biol. 2000 Oct;68(4):511-4.

Different types of FCgamma-receptors are involved in anti-Lewis Y antibody induced effector functions in vitro.
M.Dettke, H.Loibner.
Br J Cancer 82, 441-445 (2000)

A double blind randomized trial comparing immunization with anti-idiotype goat antibody vaccine SCV 106 versus unspecific goat antibodies in patients with metastatic colorectal cancer
H.Samonigg, M.Wilders-Truschnig, I.Kuss, R.Plot, H.Stöger, M.Schmid, T.Bauernhofer, A.Tiran, T.Pieber, L.Havelec and H.Loibner
J. Immunother 22, 481-488 (1999)

Humanized Anti-Lewis Y Antibodies: In Vitro Properties and Pharmacokinetics in Rhesus Monkeys
M.S.Co, J.Baker, K.Bednarik, E.Janzek, W.Neruda, P.Mayer, R.Plot, B.Stumper, M.Casquez, Cary Queen and H.Loibner
Cancer Research 56, 1118-1125 (1996)

Reduction of metastatic carcinoma cells in bone marrow by intravenously administered monoclonal antibody: Towards a novel surrogate marker for monitoring adjuvant therapies of solid tumors
G.Schlimok, K.Pantel, H.Loibner, I.Fackler-Schwalbe, G.Riethmüller
Eur. J. Cancer 31A, 1799-1803 (1995)

Enzymatic synthesis of analogs of bacterial lipid A and design of biologically active LPS-antagonists and -mimetics.
M. Bulusu, H.Hildebrandt, C.Lam, E.Liehl, H.Loibner, I.Macher, D.Scholz, E.Schütze, P.Stütz, H.Vyplel, F.Unger
Pure and Applied Chemistry 66, 2171-2174 (1994);

Enhancement of retroviral infection in vitro by anti-LeY IgG: Reversal by humanization of monoclonal mouse antibody
J.E.Hansen, A.M.Sorensen, M.Arendrup, C.Nielsen, S.Oloffson, J.O.Nielsen, E.Janzek, H.Loibner
APMIS 101, 711-718 (1993)

Model for measurement of micrometastasis in epithelial tumours
G.Schlimok, K.Pantel, F.Lindemann, H.Loibner, G.Riethmüller
In: Hemopoietic Growth Factors and Mononuclear Phagocytes; Van Furth R. (ed.), pp 168-176 (1993)

Immune response to tumor antigens in a patient with colorectal cancer after immunization with anti-idiotype antibody
H.Samonigg, M.Wilders-Truschnig, H.Loibner, R.Plot, A.Rot, I.Kuss, G.Werner, H.Stöger, M.Wrann, D.Herlyn, H.Koprowski
Clin. Immunol. and Immunopath. 65, 271-277 (1992)

Enzymatic synthesis and comparative biological evaluation of a phosphonate analog of the lipid A precursor
D.Scholz, K.Bednarik, G.Ehn, W.Neruda, E.Janzek, H.Loibner, K.Briner and A.Vasella
J. Med. Chemistry 35, 2070-2074 (1992)

Phase I/II study of monoclonal antibody against Lewis Y hapten in relapsed small cell lung cancer
R.A.Stahel, H.Lacroix, J.P.Sculier, R.Morant, J.Richner, E.Janzek, H.Loibner and H.Blythman
Ann. of Oncology 3, 319-320 (1992)

Synthesis of fluorinated analogues of Lipid A
H.Vyplel, D.Scholz, H.Loibner, M.Kern, K.Bednarik and H.Schaller
Tetrahedron Letters 33, 1261-1264 (1992)

Polyclonal anti-idiotypic antibodies mimicking the small cell lung carcinoma antigen cluster-5A interact with a panel of antibodies and induce specific immune response in animals
C.Zwicky, R.A.Stahel, H.Jaksche, R.Waibel, H.P.Lehmann and H.Loibner
Brit. J. Cancer 63, Suppl. XIV, 67-70 (1991)

Biological activity in the human system of isotype variants of oligosaccharide Y specific murine Mabs
D.Scholz, M.Lubeck, H.Loibner, J.McDonald-Smith, Y.Kimoto, H.Koprowski and Z.Steplewski
Cancer Immunol. Immunther. 33, 153-157 (1991)

Tumor cell lysis and tumor growth inhibition by the isotype variants of Mab BR55-2 directed against Y oligosaccharide
Z.Steplewski, M.Lubeck, D.Scholz, H.Loibner, J.McDonald Smith and H.Koprowski
In Vivo 5, 79-84 (1991)

Treatment of a colon carcinoma patient with SDZ SCV 106 (anti-id 17-1A), a case study
H.Samonigg, H.Loibner, M.Wilders-Truschnig, R.Plot, H.P.Brezinschek, A.Rot, I.Kuss, G.Werner, H.Stöger, M.Wrann, M.Schmid, G.H.Schneider, K.Arian-Schad, M.Klimpfinger, D.Herlyn and H.Koprowski
Proceedings of an International Symposium organized by the Department of Internal Medicine, Barmherzige Brüder Eggenberg Hospital, Graz, March 16, 1990; 1991 in: Modern aspects of tumor diagnosis and treatment. O.Eber, P.Lind, W.Langsteger (Eds.)

Immunoreactivity of patient with colorectal cancer metastasis after immunization with antiidiotypes
H.Loibner, R.Plot, A.Rot, G.Werner, M.Wrann, H.Samonigg, M.Schmid, H.Stöger, M.Truschnigg, D.Herlyn et al.
Lancet 335, 171 (1990)

Oligosaccharide Y specific monoclonal antibody and its isotype switch variants
Z.Steplewski, M.Blaszczyk-Thurin, M.Lubeck, H.Loibner, D.Scholz, H.Koprowski
Hybridoma 9, 201-210 (1990)

Chemical synthesis of endotoxin analogs and some structure activity relationships
P.L.Stütz, H.Aschauer, J.Hildebrandt, C.Lam, H.Loibner, I.Macher, D.Scholz, E.Schütze, H.Vyplel
Int. Congr.Ser.- Excerpta Med. 923, In: Cell. Mol. Aspects Endotoxin React. p129-144, Eds.: Nowotny A., Spitzer J., Ziegler E., Amsterdam, Elsevier (1990)

Selective one step synthesis of N-acetylated polyamines and a HPLC-system for their analytical separation
H.Loibner, G.Seidl
Recent Progress in Polyamine Research, 1985; L.Selmeci, M.E.Brosnan, N.Seiler (Eds.), p597-605 (1985)

Reductive methylation of primary and secondary amines with formaldehyde and phosphorous acid salts
H.Loibner, A.Pruckner, A.Stütz
Tetrahedron Lett. 25, 2535-2536 (1984)

Synthesis and structure/activity relationships of new guanidino derivatives of aminoglycoside antibiotics
W.Streicher, H.Loibner, J.Hildebrandt, F.Turnowsky
Drugs Exp. Clin. Res. 9, 591-598 (1983)

Role of the 1-amino group in aminocyclitol antibiotics: Synthesis of 1-deaminogentamicin C2
M.Philippe, B.Quiclet-Sire, A.M.Sepulchre, S.D.Gero, H.Loibner, W.Streicher, P.Stütz, N.Moreau
J.Antibiot. 36, 250-255 (1983)

1-N-Acylation of gentamicin C1a by a cyclic, chiral gamma-amino-alpha-hydroxy acid related to the (S)-4-amino-2-hydroxybutyric acid
M.Philippe, A.M.Sepulchre, S.D.Gero, H.Loibner, W.Streicher, P.Stütz
J. Antibiot. 35, 1507-1512 (1982)

A low-cost medium-pressure liquid chromatography system for preparative separations
H.Loibner, G.Seidl
Chromatographia 12, 600-604 (1979)

Synthesis of 3-deoxy-3-aminovitamin D3 and 3-deoxy-3-epiamino-vitamin D3 and D2
H.Loibner, E.Zbiral
Tetrahedron Lett. 34, 713-716 (1978)

Reactions with organophosphorus compounds, XLIII. Structural modifications of nucleosides by means of triphenylphosphane/diethyl azodicarboxylate
H.Loibner, E.Zbiral
Justus Liebigs Ann. Chem. 1, 78-86 (1978)

Reactions using triphenylphosphane/azodicarboxylate. 2. Reactions with organophosphorus compounds. XLII. Nucleophilic substitution reactions of hydroxysteroids using triphenylphosphane/diethylazodicarboxylate
H.Loibner, E.Zbiral; Helv. Chim. Acta 60, 417-425 (1977)
Reactions with organophosphorus compounds. XLI. New synthetic aspects of the triphenylphosphine-diethyl azodicarboxylate-hydroxy compound system
H.Loibner, E.Zbiral
Helv. Chim. Acta 59, 2100-2113 (1976)

Poster and oral presentations (1990-1998)

Phase I trial of cancer vaccine IGN101 in patients with epithelial cancers
H.Samonigg, G.Hofmann, S.Schagerl, G.Himmler, T.Putz, H.Loibner
Proceedings of ASCO Vol. 21, 2002, abstract 1898

Reduction of EpCAM positive cells in per9ipheral blood of patients with epithelial cancers following vaccination with the cancer vaccine IGN101
H.Loibner, G.Himmler, T.Putz, B.Peball, G.Hofmann, S.Schagerl, H.Samonigg
Proceedings of ASCO Vol. 21, 2002, abstract 1899

Sequential affinity purification of antibodies induced by vaccination with MMA383: quantitative and qualitative characterization
E.Wasserbauer, G.Ehn, R.Plot, F.Süssenbacher, A.Neubauer, W.Neruda and H.Loibner
Poster, ISPPP '98, Nov. 1 - 4, 1998, Vienna

MMA 383: Anti-idiotypic antibody mimicking the Lewis Y carbohydrate for vaccination against epithelial cancer
H.Loibner, R.Plot, E.Wasserbauer, G.Ehn, P.Mayer, E.Janzek, W.Neruda, C.Rossbacher, W.Kinzy
Poster, Symposium Antibodies –Molecular, Cellular and Clinical Aspects, April 22-24, 1998 NewYork City

Anti-Lewis Y cancer vaccine MMA 383,
H. Loibner, C. Smith
Lecture, BTDG Meeting Sept. 1997, Vienna

Charge microheterogeneity of a murine monoclonal IgG1 anti-idiotypic antibody
G.Waxenecker, E.Wasserbauer, H.Katinger and H.Loibner
Lecture, 45th Interlaken Conference, March 1997, Switzerland

Separation of a murine monoclonal IgG1 anti-idiotypic antibody in isoforms by multicompartment electrophoresis
G.Waxenecker, E.Wasserbauer, H.Katinger and H.Loibner
Lecture, 45th Interlaken Conference, March 1997, Switzerland

Monoklonale Anikörper und anti-idiotypische Antikörp r in der Krebstherapie
H.Loibner
Lecture, CEIP-Continuing Education in Industrial Pharmacy , Basel, 28.11.95

Expression of the Lewis Y oligosaccharide, a blood group-related difucosyl lacto type II series antigen, on human myeloleukemic cell lines and mature peripheral neutrophils
M.Dettke, G.Palfi, E.Rüde, H.Loibner
Poster, Jahrestagung ESCI, Cambridge, UK, April 1995

Immunization with anti-idiotype vaccine SCV 106 prolongs survival time of cancer patients with metastaticcolorectal cancer: A randomized double blind clinical trial
H.Samonigg, M.Wilders-Truschnig, I.Kuss, R.Plot, H.Stöger, M.Schmid, T.Bauernhofer, A.Tiran, L.Havelec and H.Loibner
Poster, Jahrestagung d. Deutschen und Österreichischen Gesellschaft f. Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.125, Abstr. 498 (1994);
Lecture, Third International Symposium Biological Therapy of Cancer , April 19-22, 1995, Munich, Germany

Vaccination of rhesus monkeys with an anti-idiotype antibody mimicking Lewis Y: Enhancement of the anti-tumor immune response by repeated administrations of rhGM-CSF
H.Loibner, E.Janzek, B.Stumper, P.Mayer, E.Liehl
Poster, Jahrestagung d. Deutschen und Österreichischen Gesellschaft f. Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.92, Abstr. 365 (1994); and
Poster, 3rd International Symposium Biological Therapy of Cancer , Munich, 19-22 April 1995

Individual metastatic carinoma cells in bone marrow of cancer patients are depleted by treatment with a monoclonal antibody directed against Lewis Y
G.Schlimok, G.Riethmüller, K.Pantel, I.Fackler-Schwalbe, H.Loibner
Poster, Jahrestagung d. Deutschen und Österreichischen Gesellschaft f. Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.129, Abstr. 513 (1994)

Phase II study of a multiple dose intravenous administration of a murine monoclonal IgG3 antibody (SDZ ABL 364) in brest carcinoma patients with low tumor burden
I.Kuss, H.Samonigg, H.Stöger, A.-K.Kasparek, M.Schmid, T.Bauernhofer, R.Moser, F.Ploner, E.Derstvenscheg, C.Lackner, M.Wilders-Truschnig, R.Plot, H.Loibner
Poster presentation, Jahrestagung der Deutschen und Österreichischen Gesellschaft für Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.86, Abstr. 341 (1994)

A high molecular weight glycoprotein bearing blood group related carbohydrate antigens of the Lewis family (LeX; LeY) is shed by epithelial tumor cells in vitro and in vivo
M.Dettke, F.Süssenbacher, E.Wasserbauer, E.Rüde, H.Loibner
Poster presentation, Jahrestagung der Deutschen und Österreichischen Gesellschaft für Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.24, Abstr. 95 (1994); and
Annual Meeting of the European Society for Clinical Investigations, Toledo, April 1994

Humanized anti-Lewis Y antibody - in vitro properties and pharmacokinetics in rhesus monkeys
H.Loibner, K.Bednarik, E.Janzek, W.Neruda, P.Mayer, M.S.Co
Poster presentation, Jahrestagung der Deutschen und Österreichischen Gesellschaft für Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.92, Abstr. 368 (1994); and SBT 1993, Nashville, Tennessee, Abstracts of the Society for Biological Therapy, Annual Meeting, J. Immunother. 14, p358 (1993)

A monoclonal anti-idiotypic antibody that mimics the Lewis Y carbohydrate antigen - crossreactivity with a CDR-grafted version of Ab1 as proof of internal image properties
H.Loibner, H.Jaksche, R.Plot, E.Pursch and E.Janzek
Poster presentation, Jahrestagung der Deutschen und Österreichischen Gesellschaft für Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.92, Abstr. 366 (1994); and SBT 1993, Nashville, Tennessee, Abstracts of the Society for Biological Therapy, Annual Meeting, J. Immunother. 14, p357 (1993)

A monoclonal anti-idiotypic antibody that mimics the Lewis Y carbohydrate antigen - immunization of rhesus monkeys leads to a strong immune response specific for Lewis Y positive cancer
H.Loibner, H.Jaksche, E.Janzek, M.Attinger, M.Azria
Poster presentation, Jahrestagung der Deutschen und Österreichischen Gesellschaft für Hämatologie und Onkologie, Vienna, Onkologie 17, S2, S.92, Abstr. 367 (1994); and SBT 1993, Nashville, Tennessee, Abstracts of the Society for Biological Therapy, Annual Meeting, J.Immunother. 14, p357 (1993)

Immunization of rhesus monkeys with an anti-idiotype antibody mimicking Lewis Y: Enhancement of the anti-tumor immune response by repeated administrations of rhGM-CSF
H.Loibner, P.Mayer, E.Liehl
Short oral communication, AACR 1994, April 10-13, 1994, San Francisco

A monoclonal anti-idiotypic antibody that mimics the Lewis Y carbohydrate antigen - Internal image properties and induction of a strong anti-tumor immune response in rhesus monkeys
H.Loibner, K.Bednarik, H.Jaksche, W.Neruda, R.Plot, E.Pursch, E.Janzek
Poster presentation, AACR, Nov. 7-11, 1993, Asheville, North Carolina

Real-time biospecific interaction analysis for the characterization of anti-idiotypic antibodies
H.Loibner, R.Plot, E.Janzek
Oral presentation, BIA-Symposium, Sept. 1993, London

Monoclonal Lewis Y antibody for treatment of breast cancer patients: Demonstration of depleting effect on metastatic cells in bone marrow
G.Schlimok, I.Fackler-Schwalbe, K.Pantel, H.Loibner, G.Riethmüller
Poster, ASCO 1993, May 1993, Orlando, USA
Proc. Annu. Meet. Am. Soc. Clin. Oncol. 12: A939, 1993

Properties of humanized monoclonal antibodies directed against the Lewis Y carbohydrate antigen
H.Loibner
Lecture at Scripps Research Institute, La Jolla, California, USA, March 17, 1993

Murine IgG3 anti-Lewis Y antibody SDZ ABL 364 and its mouse/human chimera stimulate the secr tion of TNF in ADCC via engagement of monocytic Fc II receptors
M. Dettke, H.Loibner
Poster, 8th International Conference on Monoclonal Antibody Immunoconjugates for Cancer, 18.-20.3.1993, San Diego, USA

Generation and characterization of humanized anti-Lewis Y antibodies
H.Loibner, K.Bednarik, E.Janzek, W.Neruda, R.Plot, M.S.Co, J.Baker
Poster, IBC Concerence, 14.-16.12.1992, San Diego, USA;
Poster, 8th International Conference on Monoclonal Antibody Immunoconjugates for Cancer, 18.-20.3.1993, San Diego, USA

Inflammation at tumor site induced by treatment of breast cancer patients with anti Lewis Y Mab SDZ ABL 364; assessment by thermographic equipment
H.Samonigg, H.Stöger, M.Wilders-Truschnig, E.Janzek, R.Plot, H.Loibner
Poster, Williamsburg Conference of Biological and Immunological Treatments of Cancer, 29.10.-1.11.1992, Williamsburg, USA; Poster, SFI, December 1992;
Poster, 8th International Conference on Monoclonal Antibody Immunoconjugates for Cancer, 18.-20.3.1993, San Diego, USA

SDZ ABL 364, a murine monoclonal IgG3 antibody directed against the Lewis Y antigen inhibits tumor cell adhesion to endothelial cells in vitro
M.Dettke, H.Loibner
Poster, Williamsburg Conference of Biological and Immunological Treatments of Cancer, 29.10.-1.11.1992, Williamsburg, USA;

Fc-dependent binding self-cooperativity of SDZ ABL 364, a murine IgG3 antitumor Mab as demonstrated by Biospecific Interaction Analysis
H.Loibner, K.Bednarik, E.janzek, W.Neruda, R.Plot
Poster, Williamsburg Conference of Biological and Immunological Treatments of Cancer, 29.10.-1.11.1992, Williamsburg, USA; and
Oral presentation, BIA-Symposium 1992, Paris, Abstr. S.34 (1992)

Anti-Lewis Y Mab SDZ ABL 364: a candidate for the treatment of minimal residual cancer
H.Loibner
Lecture, 8th International Congress of Immunology Satellite Symposium "Immunpharmacology", August 1992, Budapest

Differential immune response to the carbohydrate and peptide parts of novel synthetic tumor glycopeptide immunogen
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Invent rship or co-inventorship (US patents, EP patents and WO publications)

US5562903
HUMANIZED ANTIBODIES THAT RECOGNIZE DIFUCOSYL LEWIS BLOOD GROUP ANTIGENS Y-6 AND B-7-2

US5523085
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US4503046
1-NITRO-AMINOGLYCOSIDE DERIVATIVES, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND SUCH DERIVATIVES FOR USE AS PHARMACEUTICALS

EP1140168
USE OF ANTIBODIES FOR ANTICANCER VACCINATION

EP0644947
ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES AGAINST THE LEWIS Y-SPECIFIC MONOCLONAL ANTIBODY BR55-2 AND THEIR USES.

EP0547079
NEW USE OF A MONOCLONAL ANTIBODY.

EP0528767
ANTIBODY DERIVATIVES.

EP0445078
NEW USE OF A MONOCLONAL ANTIBODY.

EP0072351
AMINOGLYCOSIDE DERIVATIVES, PROCESSES FOR THEIR PRODUCTION, PHARMACEUTICAL COMPOSITIONS CONTAINING THEM AND SUCH DERIVATIVES FOR USE AS PHAR

EP0056408
GUANYLATED AMINOGLYCOSIDES, A PROCESS FOR THEIR PRODUCTION AND THEIR USE AS PHARMACEUTICALS.

WO0170272
POLYSACCHARIDE-POLYPEPTIDE CONJUGATE

WO0170264
METHOD FOR PRODUCING VACCINES CONTAINING A HEAT-TREATED MIXTURE CONSISTING OF AT LEAST ONE ANTIGEN AND OF AT LEAST ONE ADJUVANT

WO0135989
NOVEL USE OF ANTIBODIES AS VACCINES

WO0041722
USE OF ANTIBODIES FOR ANTICANCER VACCINATION

WO9324647
ANTI-IDIOTYPIC MONOCLONAL ANTIBODIES AGAINST THE LEWIS Y-SPECIFIC MONOCLONAL ANTIBODY BR55-2 AND THEIR USES

WO9203165
NEW USE OF A MONOCLONAL ANTIBODY

WO8401386
NEW AMINE GLUCOCYDE DERIVATIVES, PREPARATION AND UTILIZATION METHOD

WO8200464
GUANYLATED AMINOGLYCOSIDES,A PROCESS FOR THEIR PRODUCTION AND THEIR USE AS PHARMACEUTICALS

List of publication
Hans Loibner, Ph.D.
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Exhibit 3

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Detection of Murine Immunoglobulin in Human Tissues Following Therapeutic Infusion of Monoclonal Antibody

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ABSTRACT

A class switch variant of hybridoma CO19-9 secreting IgG2a antibodies was shown to have the same immunoperoxidase binding pattern in human tissue as the IgG1 antibody secreted by the parental hybridoma. The IP tissue binding of GA73.3 and 17-1A monoclonal antibodies which have been suggested to bind to structurally related antigens were compared; although quite similar in distribution, some differences were noted. GA73.3 bound to 12/12 colon carcinomas compared to 17-1A which reacted with 11/12 tumors. In several cases, the percentage of cells reactive with GA73.3 (90-100%) exceeded those reactive with 17-1A (10-25%). Additionally, the intensity of reactivity for GA73.3 was consistently greater than that seen with 17-1A. The detection of murine antibody bound to human tissues following therapeutic infusion of 19-9 IgG2a or GA73.3 differed. Detection of antibody-antigen complexes was seen less often in patients who had received 19-9 IgG2a (2/6) than in patients who had received GA73.3 (5/5). Additionally, the presence of murine immunoglobulin was seen only in the extracellular mucin of the patients receiving 19-9 IgG2a, whereas strong cellular binding of murine immunoglobulin was noted following infusion with GA73.3.

INTRODUCTION

Previous studies have shown the formation of antigen-antibody complexes in tissues after systemic infusion of murine antibody 17-1A in patients with advanced colorectal carcinoma.[1,4] These studies showed that murine antibody could be detected in tumor tissue 1-2 days following infusion, but was no longer detected 1-3 weeks following therapy.[1,4] Monoclonal antibody GA73.3 is an antibody with binding specificity to various human tumors and which is tumoricidal in vitro and in vivo in nude mice.[3] For this reason it was selected for use in a clinical trial for patients with colorectal carcinoma. Recently anti-anti-idiotypic antibodies (Ab3) were made to the GA73.3 (Ab1).[5]

Class switch variants of the hybridoma CO19-9 have been selected and stud-

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ied for their binding specificities.[15] The IgG2a switch variant has been shown to have similar binding specificity to live colorectal carcinoma cell lines.[15]

The aim of these human tissue immunoperoxidase studies was severalfold. First, we compared the IP tissue binding of 19-9 IgG2a and the parental IgG1 of GA73.3 and 17-1A, which may detect structurally similar antigens, and of GA73.3 (Ab1) and a murine anti-anti-idiotypic antibody (Ab3). We also characterized the formation of murine antibody-antigen complexes in a new series of patients who received monoclonal antibody immunotherapeutic infusion.

MATERIALS AND METHODS

Patients

A variety of normal or malignant tissues from different sites were collected from surgical specimens. Eleven patients in an immunotherapeutic trial for advanced colorectal carcinoma had tissue removed during this trial for IP studies. The patients included 6 patients who received 19-9 IgG2a for colon carcinoma: the stage at time of presentation included 2 patients with stage B2, 1 with a C2 lesion and 3 with stage D disease. Patients received from 10 to 640 mg of antibody. Patients receiving GA73.3 had either stage C2 (2), stage D (2), colon carcinoma or advanced pancreatic carcinoma (1). Patients received from 10 to 600 mg of antibody. Details of the clinical trial are reported elsewhere.

Monoclonal antibodies

Antibody 19-9 IgG2a is a class switch variant of the IgG1 secreting parent CO19-9 isolated by sib selection[15] and has previously been shown to have similar binding specificity to live SW1116 colorectal carcinoma cell lines[16] by radioimmunoassay. Monoclonal antibody CO19-9 is directed against a monosialganglioside antigen;[2,10] distribution of CO19-9 antigen on normal and malignant tissue has been described in detail elsewhere.[1] Antibody GA73.3 (IgG2a) (referred to as Ab1) is an antibody showing specificity against a variety of human tumors and has tumoricidal activity in vitro and in vivo in nude mice.[3] Anti-anti-idiotypic antibody to the GA73.3 antibody has been made and described recently;[5] the anti-anti-idiotypic antibody (Ab3) CE5G6 is IgG1 isotype and has similar binding characteristics to human tumor cell lines and has similar binding affinity to the 30-37 Kd antigen detected by GA73.3.[5] Antibody 17-1A (IgG2a) has been described in detail elsewhere.[4,7,9,12-14] MAbs were used as supernatant fluid from cell culture grown hybridoma cells. For negative control, supernatant of myeloma P3X63Ag3 was used.

Immunoperoxidase assay (IP)

Fresh tissue specimens of colon carcinomas, metastatic colon carcinomas and adjacent normal colon or rectal mucosa were immediately immersed in Tissue Tek II (Lab Tek Products, Naperville, IL), and frozen in liquid nitrogen and stored at -70°C. Sections of 5-6 μm were cut, mounted on gelatin coated slides and the procedure performed as previously described.[16] Briefly, the sections were fixed in 10% neutral phosphate buffered formalin for 10 min, washed in PBS for 10 min and treated with diluted normal horse serum. Sections were incubated with undiluted tissue culture supernatants containing approximately 4-10 μg/ml of antibody for 30 min. Sections were then incubated sequentially with biotinylated inylated horse anti-mouse IgG antibody and an avidin-biotinylated peroxidase complex according to standard recommendation of the vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA). Diamino-benzidine 0.06% in PBS containing 0.01% H_2O_2 was used as a chromagen. In order to detect binding of infused murine immunoglobulin the IP procedure was performed as above, except that the primary murine antibody was omitted and the sections were incubated with PBS instead. Thus only those sections that had bound murine antibody showed IP staining.[16] Sections were counterstained with hematoxylin, dehydrated and mounted with coverslips.

RESULTS

Comparison of immunoreactivity in tissue of therapeutic MAb

Parallel sections of normal and malignant tissues were tested with either 19-9 IgG2a or the IgG1 antibody secreted by the parental hybridoma CO19-9. The binding reactivity of the two antibodies were indistinguishable; these results are summarized in Table 1. In normal colon the antibodies bound to 5-100% of colonic cells. Binding was seen in nongoblet colonic cells only. Strong staining was also noted in bronchial epithelium and submosal glands as well as pancreatic ducts. Binding was not seen in the kidney or bone marrow. As in normal tissues, the binding patterns of 19-9 IgG2a and 19-9 IgG1 antibodies in tumors were similar with maximal expression in adenocarcinomas. The percentage of reactive cells varied from 5 to 100%; in two patients with colon carcinoma, binding was seen predominantly in the mucin and necrotic material associated with the malignant cells. Reactivity in viable tumor cell was seen in less than 5% of the cells in these two patients.

The binding of GA73.3 and anti-colorectal carcinoma antibody 17-1A were compared in a series of frozen tissues from the colon. GA73.3 bound strongly to 12/12 colon carcinomas compared to 17-1A, which bound to 11/12 colon carcinomas (Table 2). In several cases, the percentage of cells showing reactivity differed significantly with GA73.3 binding to 90-100% of the tumor cells and 17-1A with 10-25% of the cells. The degree of intensity was also different. Staining with GA73.3 consistently resulted in a strong ++ reaction, whereas staining with 17-1A gave a + reaction. In one patient, 17-1A was undetectable, whereas GA73.3 was seen in 100% of the tumor and normal cells. A difference in intensity of reactivity and % of reactive cells with the two antibodies interestingly was not as often seen in normal colonic mucosa (Table 2). Serial sections of normal and malignant tissue from two patients were tested with GA73.3 (IgG2a) and the mon-

TABLE 1. COMPARISON OF IMMUNOREACTIVITY USING IP ASSAY

Tissues	19-9 γ2a	19-9 γ1
Normal and adj. normal		
Kidney	0/4	0/4
Bone marrow	0/2	0/2
Colon	5/8	5/8
Stomach	0/5	0/5
Lung	1/1	1/1
Pancreas	5/6	5/6
Carcinomas		
Colon	8/11	8/11
Stomach	2/2	2/2
Pancreas	2/2	2/2
Lung	1/1	1/1

TABLE 2. COMPARISON OF IMMUNOREACTIVITY IN FROZEN SECTIONS OF NORMAL AND MALIGNANT COLONIC MUCOSA FOR GA 73-3 and 17-1A

Patient	Normal*		Tumor*	
	GA 73-3	17-1A	GA 73-3	17-1A
1	++ 100	0	++ 100	0
2a	0	+ 100	–	–
2b	++ 100	+ 100	++ 100	+ 100
3	++ 100	+ 100	++ 100	+ 100
4	–	–	++ 100	++ 100
5a	–	–	++ 100	+ 80
5b	–	–	++ 100	++ 80
6	–	–	+ 90	+ 25
7	++ 100	++ 100	++ 100	++ 100
8	++ 100	++ 100	+ 100	+ w 10
9	++ 100	+ 100	++ 100	+ 50
10	++ 100	0	++ 100	+ 10
11	++ 100	+ 50 luminal	++ 100	+ w 10
12a	++ 100	++ 100	++ 100	+ 50
b	++ 100	++ 100	++ 100	++ 80

*Tissues studied were taken after therapy unless otherwise indicated.

a = before therapy; b = after therapy; – = not done; w = weak reacting.

oclonal anti-anti-idiotypic antibody CE 5G6 (IgG1). In frozen tissue the binding reactivity of the two antibodies was indistinguishable, whereas IP staining was not detectable using CE 5G6 on fixed tissue from the same patient (Table 3).

Detection of murine immunoglobulin in tissues following therapeutic infusion

Tissue removed from patients having received either GA73.3 or 19-9 IgG2a antibody intravenously was tested for the presence of bound murine immunoglobulin. In some patients frozen tissue prior to infusion was also tested. Tissues were removed 1-2 days after monoclonal antibody infusion and included recurrent carcinoma, carcinoma metastatic to liver and normal rectal or colonic mucosa.

Although for either GA73.3 or 19-9 IgG2a MAb patients received comparable amounts of antibody (10-640 mg), there were striking differences in the detection of bound murine immunoglobulin following infusion. In patients who had received 19-9 IgG2a, murine Ig was detected infrequently (2/6) (Table 4). Evidence for bound murine immunoglobulin was seen in only two patients. Both patient E and patient F received higher amounts of antibody (300 mg and 640 mg respectively). Murine antibody antigen complexes were detected in the extracellular mucin and necrosis surrounding the tumor only. Evidence for bound murine immunoglobulin was the presence of diffuse + staining seen with PBS as the

TABLE 3. COMPARISON OF Ab1 AND Ab3 IMMUNOREACTIVITY IN COLONIC TISSUE

	Ab1 GA73.3	Ab3 CE 5G6
Normal		
Frozen	2/2*	2/2*
Fixed	2/2	0/2
Carcinoma		
Frozen	2/2	2/2
Fixed	2/2	0/2

*100% of nongoblet cells reactive.

TABLE 4. DETECTION OF MURINE Ig IN TISSUES FOLLOWING INFUSION OF 19-9 IgG2a ANTIBODY

Patient	Amount of MAb Infused	Tissue Tested	Pre-MAb Infusion	Post-MAb Infusion*
A	10 mg	Normal		0
		Carcinoma		0
B	10 mg	Normal	0	0
		Carcinoma	0	0
		Metastasis	0	0
C	30 mg	Normal	0	0
		Carcinoma	0	0
D	100 mg	Normal		0
		Carcinoma		0
E	300 mg	Carcinoma	0	+**
F	640 mg	Carcinoma	0	+**

*Results given as insensity of reactivity and percentage of reactive cells using PBS as initial incubator to detect bound murine immunoglobulin (see Materials and Methods).

**Mucin only.

initial incubation followed sequentially by biotinylated anti-murine immunoglobulin and the avidin/biotin/peroxidase complex. Sections of the tumors of these patients showed less than 5% cells reactions with either 19-9 IgG1 or 19-9 IgG2a. In the other patients however, there was staining for GICA with either antibody in the carcinoma, but there was no evidence for bound murine Ig following infusion. The lack of murine antibody-antigen complexes, therefore, does not necessarily correlate with lack of antigen in the tissue. GICA was seen in both normal and tumor cells of patients A, C and D, but only in the carcinoma of

TABLE 5. DETECTION OF MURINE Ig IN TISSUES FOLLOWING INFUSION OF GA73.3 ANTIBODY

Patient	Amount of MAb Infused	Tissue Tested	Pre-MAb Infusion	Post-MAb Infusion*,**
A	10 mg	Normal		0
		Carcinoma		++ 10
		Metastasis		++ 5
B	30 mg	Metastasis		+ edge of tumor
C	100 mg	Normal	0	
		Carcinoma	0	++ 60
		Metasasis		++ 40
D	300 mg	Normal		++ 100
		Carcinoma§		++ 100
E	600 mg	Normal		++ 100
		Carcinoma		++ 100

*Results given as intensity of reaction and percentage of reactive cells with PBS as initial incubation (see Materials and Methods).

**Tissues removed 1-2 days after infusion of monoclonal antibody.

§Pancreatic carcinoma and normal tissue.

patient B (data not shown). On the other hand, 5/5 patients who received GA73.3 had evidence for murine antibody bound to post infusion tissue and staining was seen intracellularly. Staining was seen in both normal and tumor tissues (Table 5). With increasing amounts of administered antibody greater percentage of cells showed the presence of bound murine immunoglobulin so that in patient A, who received 100 mg of GA73.3, only 10% of the carcinoma and 5% of a concurrent liver metastasis showed staining with PBS as the initial incubation step. Additionally, in patient B in whom 30 mg was given, only the edge of the tumor metastasis showed murine antibody-antigen complexes. In contrast, patient D, who had a pancreatic carcinoma and patient E with a colon primary, 100% of the normal and malignant cells showed evidence for bound murine immunoglobulin 1-2 days following infusion of antibody.

DISCUSSION

Although 19-9 IgG2a shows binding to sections of normal bronchial mucosa and colonic epithelium, there were no symptoms referable to these sites following therapeutic infusion of this antibody. This is similar to our experience with 17-1A antibody which has been shown to bind to sections of normal renal tubules, bronchial epithelium and colonic mucosa.[12-14] It has been our experience that for selection of an antibody as an immunotherapeutic agent, it is reassuring to show lack of binding to normal tissues, especially bone marrow. On the other hand, binding to sections of some epithelial tissues does not correlate with nor predict symptomology of subsequently infused antibody. Binding to sections of some tissues (especially regenerative epithelium) therefore would not preclude therapeutic usefulness of some monoclonal antibodies.

We have also shown that the binding of several related antibodies is quite similar. The binding of an IgG2a class switch variant was indistinguishable from that of IgG1 antibody produced by the parental 19-9 hybridoma. This is confirmation of the binding similarities these antibodies share.[15] Similarly GA73.3 (Ab1) and an anti-anti-idiotypic antibody (Ab3) produced in mice have the same distribution as seen by IP in a small number of colon tissues. Whereas GA73.3 antibody bound to Bowin's fixed tissues from the same patients (both normal and carcinoma), IP staining was not seen in fixed sections using Ab3 anti-anti-idiotypic antibody. These IP differences suggest a difference in affinity. A similar difference in affinity may account for the differences in binding of GA73.3 and 17-1A to colonic carcinomas. Whereas GA73.3 consistently bound to 90-100% of colon carcinoma cells, the 17-1A antibody frequently bound to only 10-25% of the tumor cells.

In addition to characterizing tissue distribution of monoclonal antibody defined antigens, tissue immunoperoxidase studies play an important role in evaluating modulation of antigen with therapy, of correlating presence or absence of antigen with clinical responsiveness and for characterizing the presence of bound murine immunoglobulin following infusion of antibody.

We were able to demonstrate the presence of murine immunoglobulin on human tissue following therapeutic administration of monoclonal antibodies 19-9 IgG2a or GA73.3. There were, however, clear differences in the location and frequency of murine antibody-antigen complexes. These differences could not be related to the presence or absence of the antigen in the tissue of the patient. In patients who received 19-9 IgG2a murine immunoglobulin was detected infrequently (2/6) and was seen predominantly in the extracellular mucin surrounding the tumor cells. There is evidence that 19-9 antigen may be associated with a large mucin molecule in the serum and the monosialoganglioside detected by 19-9 has been demonstrated by IP in various mucin producing epithelia. It is possible that infused 19-9 IgG2a may have difficulty reaching viable tumor cells in lesions which produce a large amount of extracellular mucin because of strong binding to these substances. In contrast, patients who received GA73.3 showed the presence of murine antibody in all cases (5/5). A similar binding of murine immunoglobulins in tissues of patients receiving 17-1A antibody has been previously cited.[14] It is not clear if there is any relationship between the ability to detect murine antibody-antigen complexes and other similarities suggested for GA73.3 and 17-1A antibodies.

ACKNOWLEDGMENTS

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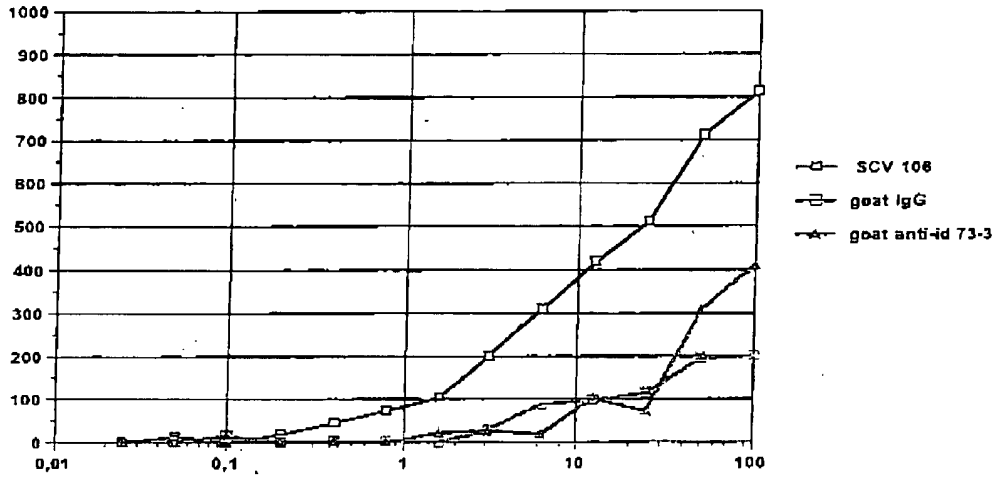
D claration of HANS LOIBNER
Exhibit 4



Binding of SCV 106, goat anti-id 73-3 and goat IgG to KATO gastric cancer cell line (cell-ELISA)

Exhibit 5

[CANCER RESEARCH 44, 681–687, February 19

Antigens Associated with a Human Lung Adenocarcinoma Defined by Monoclonal Antibodies[1]

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ABSTRACT

Monoclonal antibodies KS1/4, KS1/9, and KS1/17 were developed in this laboratory from a fusion of the murine myeloma cell line P3X63Ag8 with spleens of BALB/c mice previously primed with UCLA P3 cells derived from a human adenocarcinoma of the lung. Monoclonal antibodies KS1/4 and KS1/17 seemed to recognize similar glycoprotein antigens on the lung carcinoma cells by indirect immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. However, mapping of [^{3}H]lysine- and [^{3}H]arginine-labeled tryptic peptides of antigens in specific immunoprecipitates of lung carcinoma cells by high-pressure liquid chromatography revealed a one peptide difference. Antibody KS1/9 did not immunoprecipitate any identifiable protein from detergent extracts of the immunizing cell line by routine methods and appears to detect a glycolipid antigen. Immunocytochemical analysis of tissue sections showed this monoclonal antibody to be reactive with adenocarcinomas of the lung and not with the other histological types of lung carcinoma or normal tissue. Monoclonal antibodies KS1/4 and KS1/17, however, reacted with 3 major histological types of lung cancer and minimally with the proximal tubules of normal kidney and the epithelium of bronchioles.

INTRODUCTION

Lung cancer is now considered to be the leading cause of cancer death in both men and women with more than 120,000 new cases diagnosed each year (20). This disease, which can be divided into 4 major histological types, i.e., epidermoid (30%), adenocarcinoma (35%), large-cell undifferentiated (15%), and small-cell (20%), has responded relatively poorly to most regimens of chemotherapy, and radiation therapy and surgery appear to be the only effective therapy. However, unfortunately, fewer than 30% of lung cancer patients have tumors which can be totally resected at diagnosis and of these, fewer than one-third survive 5 years after apparent complete surgical removal of all tumor (20).

The use of immunological approaches to manipulate the immune response of lung cancer patients offers an alternative means for diagnosis, prognosis, and therapy. Surface antigens on lung tumor cells may provide the key to such an approach, since antibodies to such cell surface structures with sufficient specificity for human lung cancer could provide potentially important diagnostic and therapeutic tools. The development of hybridoma technology for the production of MoAbs[3] (10) has facilitated a new and incisive approach to produce specific molecular probes for cell surface analyses, and several investigators have reported antibodies with varying degrees of specificity for small-cell and squamous carcinoma of the lung (1, 3–6, 9, 12, 15).

In this regard, Mazauric *et al.* (12) showed that a panel of MoAbs directed against the bronchogenic carcinoma cell line MBA 9812 and the squamous carcinoma of the lung cell line WL 1680 were reactive against a number of lung carcinomas but negative against fibroblast cell lines and peripheral blood cells. Brenner *et al.* (4) reported MoAbs that were highly specific for squamous cell carcinoma, using as immunogens partially purified tumor antigens that were structurally similar to human histocompatibility antigens. Cuttitta *et al.* (6) produced MoAbs by immunizing mice with human lung cancer cells and obtained 3 hybridomas that reacted with small-cell carcinoma but not with a number of normal tissues.

Here, we report the production of a panel of MoAbs by immunizing BALB/c mice with the human adenocarcinoma of the lung cell line UCLA-P3. Two of these antibodies, KS1/4 and KS1/17, isolated from cloned and stable hybrids, were found to specifically react in ELISA assays and immunoperoxidase t sts with tumors of 3 major histological types of lung cancer. A third antibody, KS1/9, reacted with adenocarcinoma of the lung, colon, stomach, and melanoma.

MATERIALS AND METHODS

Cells

Lung tumor cell lines and lymphoblastoid cell lines were cultured in Roswell Park Memorial Institute 1640 media containing 10% fetal calf serum, 2 mM glutamine, and gentamicin (25 μg/ml). We originally obtained the lung tumor cell line T-291 (adenocarcinoma) from Dr. Masue and Dr. Sato, University of California at San Diego; the lung adenocarcinoma cell line UCLA-P3, from Dr. D. L. Morton, UCLA; and the oat cell carcinoma line NCI-H69, from Dr. John Minna, National Cancer Institute. The B-lymphoblastoid cell lines PL-3 and L14 were originally acquired from Dr. D. L. Morton, UCLA; the lymphoblastoid B-cell line LG-2 was obtained from Dr. R. Gatti at UCLA; Molt-4 is a T-cell line.

Hybridomas were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, and gentamicin (25 μg/ml), or as ascites in pristane-primed BALB/c mice.

Production of MoAbs

MoAbs were produced to the lung adenocarcinoma cell line UCLA-P3 by use of standard hybridoma technology (10). BALB/c mice were given injections once every week for a total of 4 weeks with 5×10^6 UCLA-P3 cells, and their splenocytes fused with the murine myeloma cell line P3Ag8X63 at Day 3 after the last injection.

Isotyping of MoAbs

The isotype of MoAbs was determined with a Litton Bionetics kit (Litton Bionetics, Kensington, MD) as described in the kit instructions.

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[3] The abbreviations used are: MoAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline, pH 7.1.

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Radiolabeling

The lung adenocarcinoma cell line UCLA-P3 was metabolically labeled with [^{3}H]leucine, [^{3}H]lysine, [^{3}H]arginine, or [^{3}H]glucosamine as described previously (17). Labeled cells were washed with 0.9% NaCl solution and extracted with 0.01 M Tris-HCl, pH 8.5, containing 0.15 M NaCl, 1% Renex-30, and 0.5 mM phenylmethylsulfonyl fluoride for 30 min at 4°. Following centrifugation at 15,000 × *g* for 30 min to remove insoluble material, the radiolabeled supernatant extract was stored at −20° until further use.

Indirect Immunoprecipitation and SDS-PAGE

Radiolabeled extracts of UCLA-P3 cells were reacted with MoAb bound to protein A-Sepharose or with an appropriate second antibody as described previously by Quaranta *et al.* (14). Bound antigens were analyzed by SDS-PAGE as described by Laemmli (11) and visualized by fluorography as described by Bonner and Laskey (2).

ELISA Assay

Initial specificity screening of hybridoma supernatants was by an ELISA assay using dneo cells as targets as described previously by Harper and Orengo (8). MoAb supernatants were used diluted 1:2 in 0.1% bovine serum albumin in PBS, pH 7.1, with 0.2% Tween 20 (PBS-Tween).

Inhibition ELISA Assay for Detection of Antigens in Spent Medium

MoAbs were serially diluted in 0.1% bovine serum albumin in PBS-Tween and assayed for reactivity with UCLA-P3 cells dried onto the surfaces of 96-well microliter plates using the ELISA method. The optimal dilution of MoAb was determined to be 0.01 μg/ml. Antibody at this dilution was then incubated for 2 hr at 4° with serial dilutions of spent medium from UCLA-P3 cells and also with known dilutions of purified glycoprotein extracted from these same cells with Renex 30 (19). After incubation, the resultant mixture was applied to UCLA-P3 cells dried onto 96-well plates, and the ELISA assay was performed as described.

Tryptic Peptide Mapping by High-Pressure Liquid Chromatography

The procedure used for tryptic peptide mapping is that described by Walker *et al.* (18). Briefly, antigens isolated by indirect immunoprecipitation from cell extracts labeled with [^{3}H]lysine and [^{3}H]arginine were subjected to SDS-PAGE in tube gels. These gels were sliced in 2-mm sections and the sections eluted in H_2O. The position of the peaks was determined by scintillation counting of aliquots, and the peak fractions were pooled. Human γ-globulin was added as a carrier, and the mixture was reduced with dithiothreitol and carboxamidomethylated. Proteins were precipitated with acetone, digested with L-(tosylamido-2-phenyl)ethylchloromethyl ketone-treated trypsin (Worthington Biochemical Co.), and separated by high-pressure liquid chromatography on a BioRad ODS-5S column with a complex, nonlinear gradient of 0.1 M sodium phosphate, pH 2.1, to 80% acetonitrile over a period of 90 min. Fractions were collected, dried, and counted.

Tissues

Portions of fresh normal and malignant tissue were obtained from the surgical pathology department of the Ida M. Green Hospital of Scripps Clinic. Specimens were embedded in Tissue Tek Medium (Scientific Products) and frozen in blocks in isopentane at liquid nitrogen temperature. They were then stored at −70°.

Immunoperoxidase Staining

Frozen Tissues. Sections of frozen tissue blocks, 4- to 6-μm thick, were cut on the microtome/cryostat, mounted on glass slides, briefly air-dried, and either stained immediately or stored at −70° in airtight boxes. An indirect immunoperoxidase assay, as described by Taylor (16), was used to stain these slides. Briefly, after a washing in PBS, the sections were preincubated for 15 min at room temperature in PBS containing 10% goat serum and 0.1% bovine serum albumin. Excess serum was then pipeted off, and appropriately diluted MoAb was overlayed onto the sections. Antibodies were titered for the highest dilution reacting with lung carcinoma cells without a decrease in staining intensity, and that dilution was used to screen normal tissues, e.g., for KS1/4, a 1:64 dilution of purified antibody was used; and for KS1/9, a 1:16 dilution of purified antibody was used. It was necessary to dilute the MoAbs to reduce nonspecific binding which was also detected with P3X63 supernatants. This nonspecific binding was most prominent on the mucosa of normal colon. The slides were allowed to incubate in a humid chamber for 1 hr. The sections were then briefly washed in PBS and overlaid with a 1:50 dilution of peroxidase-conjugated goat anti-mouse antibody (IgG + IgM) (Tago Chemicals). This step was followed by a 1-hr incubation at room temperature followed by a wash in PBS. The color reaction was developed with diaminobenzidine (1 mg/ml) and 0.03% H_2O_2. After a brief counterstain with 1% methylene blue, the slides were washed in water and dehydrated in isopropyl alcohol. They were then cleared in xylene, mounted in Permount, coverslipped, and examined using an American Optical Microstar Series 20 microscope.

Immunofluorescence Staining

Live tissue culture cells (1 × 10^6) of the adenocarcinoma cell line used for immunization were washed with PBS in Eppendorf tubes. Undiluted MoAb was incubated for 45 min on ice, and the cells were washed and incubated with fluorescein-labeled goat anti-mouse antibody (Tago Chemicals) for 45 min on ice. The cells were washed again and fixed using 1% formaldehyde. A small drop was applied to glass slides, coverslipped, and viewed using a Leitz Orthoplan fluorescence microscope.

RESULTS

Production and Characterization of MoAbs. Fusion of BALB/c splenocytes immunized with UCLA-P3 cells resulted in 900 hybridomas. Out of these 900 clones, 17 hybridomas were found by ELISA assay to react with lung adenocarcinoma or other tumor cell lines but not with the B-lymphoid cell line autologous with UCLA-P3, PL3, other T- and B-lymphoblastoid cell lines or the human fibroblast cell line, WI38. The ELISA binding results are summarized in Table 1. Preliminary selection by immunoperoxidase staining of tumor and normal tissues (data not shown) demonstrated that 3 of the MoAbs, KS1/4 (IgG2a), KS1/9 (IgM), and KS1/17 (IgG2b), reacted specifically with tumor cells but with few if any normal tissues. Therefore, the antigens recognized by these antibodies were further characterized.

Table 2 summarizes the pattern of reactivity with normal and tumor tissue detected by immunoperoxidase staining of MoAbs KS1/4, KS1/17, and KS1/9. MoAbs KS1/4 and KS1/17 both reacted equally well not only with adenocarcinoma, epidermoid carcinoma, and small-cell carcinoma of the lung but also with colon, breast, and stomach adenocarcinomas. Both antibodies, however, faintly stained bronchial epithelial cells of normal lung, the proximal tubules of the kidney, as well as acinar cells of the pancreas. No specific cell type in the bronchial epithelium stained. Interestingly, both MoAbs, KS1/4 and KS1/17, reacted strongly with fetal lung, fetal proximal tubules of the kidney, and fetal colon. MoAb KS1/9 reacted with adenocarcinomas of the lung, colon, stomach, and melanoma, but failed to react with all normal tissues tested. A peroxidase antiperoxidase method was used

Table 1
Reactivity of MoAbs with tissue culture cell lines by ELISA

Antibody	Adenocarcinoma: Lung P-3	Adenocarcinoma: Lung T-291	Adenocarcinoma: Pancreas (Panc-1)	Oat cell carcinoma (NCI-H69)	Melanoma M-14	Melanoma M-21	Lymphoblastoid PL-3	Lymphoblastoid LG-2	Lymphoblastoid L-14	Lymphoblastoid Molt-14	Fibroblast WI-38
KS1/1	++[a]	++	+	−	−	−	−	−	−	−	−
KS1/2	+	+	+	−	−	−	−	−	−	−	−
KS1/3	++	++	−	−	−	−	−	−	−	−	−
KS1/4	++	++	+	+	−	−	−	−	−	−	−
KS1/5	+	+	+	−	−	−	−	−	−	−	−
KS1/6	++	++	−	−	−	+	−	−	−	−	−
KS1/7	++	+	−	−	−	−	−	−	−	−	−
KS1/8	++	++	−	−	−	−	−	−	−	−	−
KS1/9	++	++	−	−	+	+	−	−	−	−	−
KS1/10	+	+	+	−	−	−	−	−	−	−	−
KS1/11	++	−	−	−	−	−	−	−	−	−	−
KS1/12	++	++	+	−	−	−	−	−	−	−	−
KS1/13	++	++	+	−	−	−	−	−	−	−	−
KS1/14	+	++	−	−	−	−	−	−	−	−	−
KS1/15	+	+	+	−	−	+	−	−	−	−	−
KS1/16	+	+	−	−	−	−	−	−	−	−	−
KS1/17	++	++	+	+	−	−	−	−	−	−	−

[a] +, ELISA absorbance of 0.2 to 1.0; ++, ELISA absorbance of 1.0 to 2.0 or greater; −, ELISA absorbance equal to background.

Table 2
Reactivity of MoAbs by immunoperoxidase staining of tissues

Tumors (no. of specimens tested)	KS1/4	KS1/17	KS1/9
Adenocarcinoma			
Lung (6)	+[a]	+	+
Colon (3)	+	+	+
Stomach (1)	+	+	+
Breast (1)	+	+	+
Pancreas (1)	+	+	−
Epidermoid carcinoma, lung (3)	+	+	−
Small-cell carcinoma, lung (6)	+	+	−
Melanoma (4)	−	−	+
Normal			
Spleen (3)	−	−	−
Liver (3)	−	−	−
Colon (3)	−	−	−
Kidney (3)	±	±	−
Lung bronchioles (3)	±	±	−
Lung alveoli (3)	−	−	−
Pancreas (3)	±	−	−
Brain (3)	−	−	−
Fetal tissues			
Lung	+	+	−
Kidney proximal tubules	+	+	−
Colon mucosal lining cells	+	+	+ (few)

[a] +, strong staining; −, no staining; ±, faint reaction with proximal tubules of kidney, bronchiolar lung epithelium and acinar cells of the pancreas.

to confirm these findings (data not shown) (16).

Fig. 1 demonstrates the immunoperoxidase staining patterns of KS1/4, KS1/9, and KS1/17 MoAbs with an adenocarcinoma of the lung. All 3 MoAbs react intensely with the tumor cells but are unreactive with surrounding normal tissue.

Immunochemical Identification of Lung Tumor-associated Antigens. Indirect immunoprecipitation of [^{3}H]leucine-labeled detergent extracts of UCLA-P3 cells with MoAbs KS1/4, KS1/17, and KS1/9 and subsequent analysis by SDS-PAGE demonstrated that KS1/4 and KS1/17 both recognize a protein of M_r 40,000 (Fig. 2). Thus far, KS1/9 failed to react by indirect immunoprecipitation with an antigen detectable on SDS-PAGE.

To determine whether KS1/4 and KS1/17 reactive antigens are glycosylated, UCLA-P3 cells were labeled with [^{3}H]glucosamine, and the resulting extract was tested by indirect immunoprecipitation with the 2 MoAbs. As is apparent from Fig. 2, the M_r 40,000 protein recognized by KS1/4 and KS1/17 is a glycoprotein, since it is capable of incorporating [^{3}H]glucosamine.

To determine if both antigens of M_r 40,000 recognized by KS1/4 and KS1/17 were identical, their tryptic peptide profiles were compared by high-pressure liquid chromatography. The resulting peptide maps are shown in Chart 1. The top of Chart 1 depicts a comparison of the [^{3}H]arginine-labeled tryptic peptides of the M_r 40,000 antigens recognized by MoAbs KS1/4 and KS1/17. The bottom of Chart 1 shows a comparison of the [^{3}H]-lysine-labeled tryptic peptides of these 2 antigens. Since both [^{3}H]lysine and [^{3}H]arginine tryptic peptides were analyzed, it is possible to compare every peptide except the COOH-terminal peptides which would not be labeled. As is evident from the data, the 2 molecules are very closely related. There appears to be complete homology between the arginine-containing peptides, although one difference exists between the lysine-containing peptides. The antigen identified by KS1/4 has a unique peptide eluting at 32 min, and a possible difference exists between the 2 molecules as indicated by the difference in the peptides which eluted at 95 min. These data suggest that the MoAbs KS1/4 and KS1/17 react with antigens that are structurally extremely similar.

Location of Antigens Reactive with KS1/4 and KS1/9 MoAbs. To determine whether the antigens recognized by MoAbs KS1/4 and KS1/9 are surface or intracellular structures, fine UCLA-P3 adenocarcinoma cells were reacted with the 2 MoAbs, and the binding was monitored with goat anti-mouse antibody conjugated to fluorescein. As is evident in Fig. 3, the plasma membrane stains very brightly, suggesting that these 2 antigens are external membrane-associated antigens. In addition, the antigens recognized by the 2 MoAbs appear to be firmly anchored in the plasma membrane, since neither antigen could be detected in spent culture media by inhibition ELISA assays. After incubation of MoAbs KS1/4, KS1/9, and KS1/17 with serial dilutions of spent medium from UCLA-P3 cells, there was no

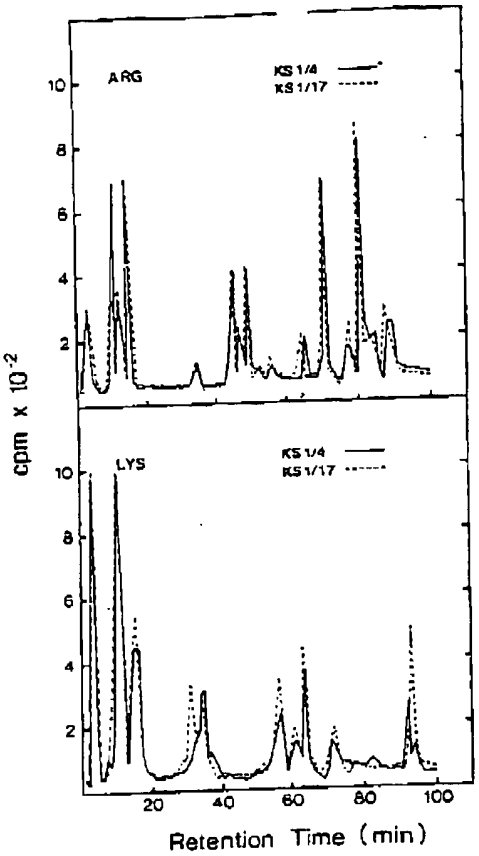

Chart 1. Tryptic peptide maps of antigens recognized by MoAbs KS1/4 and KS1/17. *Top*, comparison of the [^{3}H]arginine (*ARG*)-labeled tryptic peptides of the *M*, 40,000 antigens recognized by the 2 MoAbs; *bottom*, comparison of the [^{3}H]lysine (*LYS*)-labeled tryptic peptides of these same 2 antigens.

inhibition of reactivity with target UCLA-P3 cells. Using a purified antigen preparation, we were able to detect 2 μg of antigen per ml of spent culture medium.

DISCUSSION

It is apparent from our studies that 3 hybridomas, KS1/4, KS1/9, and KS1/17, are of interest, since they appeared to be reasonably specific for lung cancer cells by immunoperoxidase staining. When a panel of normal and tumor tissues was tested, the antibodies bound strongly to tumor tissues but either failed to react or reacted only weakly with a variety of normal tissues. It was of particular interest that MoAbs KS1/4 and KS1/17 reacted with adenocarcinoma, epidermoid carcinoma, and small-cell carcinoma, because it suggests that these 2 MoAbs may be potentially useful as diagnostic and/or therapeutic agents, since the antigen(s) recognized by them was expressed on all of the lung tumors tested.

Both MoAbs KS1/4 and KS1/17 probably react with the same or extremely similar antigen(s), since the peptide maps of the antigen recognized by the 2 MoAbs are nearly identical. While the one peptide difference detected may be due to a difference in amino acid sequence, it is equally likely that this difference may be due to a posttranslational modification such as glycosylation or phosphorylation. It also appears likely that MoAbs KS1/4 and KS1/17 react with different epitopes since, otherwise, their peptide maps would have been identical.

The origin of this *M*, 40,000 glycoprotein is unclear. It may be a new antigen expressed by lung carcinoma cell or a modified, normally expressed cell surface antigen. Because of its similarity in molecular weight with that of HLA-A,B antigens, we have compared the peptide maps of HLA-A,B antigen isolated with the MoAb W6/32 to those of the *M*, 40,000 glycoprotein and found no similarities (data not shown). From the results obtained to date, there is a strong possibility that it is a fetal antigen, since the antigen is expressed on fetal lung, kidney, and colon tissue, but is at best faintly visible on the corresponding adult tissues.

MoAb KS1/9 was found to react with lung as well as other adenocarcinomas but not with epidermoid or small-cell lung carcinomas. Since KS1/9 reacted also with melanoma, its lack of reactivity with small-cell carcinoma of the lung may be of interest, since the cell of origin of both tumors is derived from the neural crest (7, 13).

The chemical nature of the antigens recognized by KS1/9 is as yet undefined. It is apparently not a protein, since it could not be detected by indirect immunoprecipitation following labeling with [^{3}H]leucine, lysine, or glucosamine. Preliminary data (not shown) suggest that the KS1/9 MoAb binds to a lipid or glycolipid, since reactivity was detected in chloroform:methanol extracts of UCLA-P3 cells. We are currently determining the exact nature of this antigen since, thus far, it seems to react only with tumor cells and to be completely unreactive with normal tissues. The MoAbs described here appear to possess sufficient specificity for lung cancer and to be of potential diagnostic and therapeutic use, especially since preliminary experiments already suggest that, either alone or when conjugated to drugs, they can suppress the growth of human lung tumors in athymic (*nu/nu*) mice.

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Exhibit 6